Design and x-ray crystal structures of high-potency nonsteroidal glucocorticoid agonists exploiting a novel binding site on the receptor

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Crystallography and computer modeling have been used to exploit a previously unexplored channel in the glucocorticoid receptor (GR). Highly potent, nonsteroidal indazole amides showing excellent complementarity to the channel were designed with the assistance of the computational technique AlleGrow. The accuracy of the design process was demonstrated through crystallographic structural determination of the GR ligand-binding domain–agonist complex of the D-prolinamide derivative 11. The utility of the channel was further exemplified through the design of a potent phenylindazole in which structural motifs, seen to interact with the traditional GR ligand pocket, were abandoned and replaced by interactions within the new channel. Occupation of the channel was confirmed with a second GR crystal structure of this truncated D-alaninamide derivative 13. Compound 11 displays properties compatible with development as an intranasal solution formulation, whereas oral bioavailability has been demonstrated with a related truncated exemplar 14. Data with the pyrrolidinone amide 12 demonstrate the potential for further elaboration within the “meta” channel to deliver compounds with selectivity for the desired transrepressive activity of glucocorticoids. The discovery of these interactions with this important receptor offers significant opportunities for the design of novel GR modulators.

drug design | AlleGrow | glucocorticoid modulation | intranasal glucocorticoid

Since the discovery of the remarkable anti-inflammatory properties of the natural glucocorticoid cortisol 1 in the 1940s (1) this steroid template has been the subject of extensive optimization to deliver compounds with enhanced potency, selectivity, and pharmacokinetic properties (Scheme 1) (2). The most recent derivative to emerge from this effort is fluticasone furoate 2, which as Veramyst/Avamys offers highly effective once-daily treatment for both nasal and ocular symptoms of seasonal and perennial allergic rhinitis (3–4). The glucocorticoid receptor (GR) has proved to be very difficult to crystallize, but we have reported x-ray crystal structures of the glucocorticoid ligand-binding domain (LBD) containing the corticosteroid agonists dexamethasone, fluticasone propionate, and fluticasone furoate (5–7). These x-ray structures have provided a detailed picture of the interactions of steroid ligands with this important nuclear receptor.

As with other steroid receptors there has also been increasing interest in recent years in the identification of novel nonsteroidal ligands for the GR (8–12). This effort has coincided with a more detailed understanding of the mechanism of glucocorticoids (13–15). These studies have raised the prospect of even safer glucocorticoids that target the transpression (TR) activities associated with the desired anti-inflammatory activity over the transactivation (TA) activities more commonly associated with unwanted side effects of glucocorticoids. It is hoped that the structural diversity offered by nonsteroidal ligands may help deliver such “dissociated” glucocorticoids, and this has been borne out with the observation of improved side-effect profiles in preclinical models with nonsteroidal GR ligands, such as ZK 216348 3 (16) and BI-104 4 (17).

Recently we reported a series of nonsteroidal GR agonists, incorporating an aminopyrazole moiety (e.g., 5), displaying excellent selectivity for GR over other steroid hormone receptors (18, 19). Constraining the aminopyrazole amide afforded aminopyrazole derivatives (e.g., 6) showing a further increase in GR agonist potency (20). Attempts to accommodate these


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Data deposition: The atomic coordinates have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3K22 and 3K23).

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pyrazole and indazole derivatives into the ligand-binding pocket as defined by the earlier steroidal GR crystal structures were unsuccessful. However, these derivatives share structural similarities to the steroidal glucocorticoid agonist cortivazol and were found to be well accommodated in a GR binding-site model that had been developed for this extended corticosteroid (18) and that utilized side chain movements first postulated by Yoshikawa et al. (21).

This model required 2 residues (Arg-611 and Gln-570) to move substantially to accommodate the arylpyrazole moiety, and we noticed that this movement creates a new channel in the receptor extending from the meta-position of the phenyl ring. This expansion of the LBD and formation of the "meta" channel was supported by an in-house low-resolution GR crystal structure for deacetylcortivazol reported previously (18) and has recently been confirmed with a non-steroidal 17α glucocorticoid (19) and a new channel is created adjacent to the previously unexplored region of the receptor. This design work was accomplished with the aid of the computational growth procedure, AlleGrow molecular modeling system (Boston De Novo).

Results

A comparison of the recent crystal structures (7, 22) of fluticasone furoate 2 and deacetylcortivazol 8 in the GR LBD (Fig. 1A and B) illustrates how the binding pocket opens up to accommodate the extended cortivazol structure. Thus fluticasone furoate is seen to be completely enclosed by the receptor, with the partially hidden furoate ester neatly occupying the lipophilic 17α pocket on the left-hand side and the hydrogen bonds (Arg-611 and Gln-570) to the A-ring keto group defining the extremity of the binding pocket on the right-hand side (Fig. 1A). In contrast, the LBD is forced open in this direction by the phenyl pyrazole extension of the deacetylcortivazol structure, and a new channel is created adjacent to the N-phenyl group (Fig. 1B). We used the cortivazol model reported previously (18) to dock the arylindazole 6. Compound 6 shows good GR binding.

Table 1. In vitro GR and PR activities for indazoles 6 and 10–12, compared with fluticasone furoate (2)

<table>
<thead>
<tr>
<th>Compound</th>
<th>GR binding pIC50*</th>
<th>GR NF-κB pIC50 (% max)†</th>
<th>GR MMTV pEC50 (% max)†</th>
<th>PR pEC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.2 ± 0.4 (n = 12)</td>
<td>10.5 ± 0.3 (n = 57)†</td>
<td>9.9 ± 0.3 (n = 11)</td>
<td>9.1 ± 0.2 (n = 12)‡</td>
</tr>
<tr>
<td>6</td>
<td>7.7 ± 0.2 (n = 3)</td>
<td>9.7 ± 0.3 (n = 7)</td>
<td>9.1 ± 0.4 (n = 6)</td>
<td>6.8 ± 0.8 (n = 11)</td>
</tr>
<tr>
<td>10</td>
<td>8.2 ± 0.4 (n = 3)</td>
<td>10.7 ± 0.2 (n = 11)</td>
<td>9.9 ± 0.2 (n = 14)</td>
<td>8.9 ± 0.3 (n = 4)</td>
</tr>
<tr>
<td>11</td>
<td>8.2 ± 0.1 (n = 4)</td>
<td>10.3 ± 0.4 (n = 18)</td>
<td>9.6 ± 0.4 (n = 21)</td>
<td>7.7 ± 0.4 (n = 10)</td>
</tr>
<tr>
<td>12</td>
<td>Not tested</td>
<td>8.2 ± 0.1 (n = 5)</td>
<td>7.7 ± 0.3 (n = 7)</td>
<td>&lt;5</td>
</tr>
</tbody>
</table>

*Assay format A.
†Maximum response relative to dexamethasone (100%).
‡Data from Salter et al. (3).
in vitro, displays potent and full GR agonist activity in in vitro functional assays of TR and TA, and shows good selectivity over the unwanted progesterone receptor (PR) agonist activity often seen with corticosteroids (Table 1). The cortivazol model shows the same channel observed in the deacetylcortivazol crystal structure (22), and it was found that the channel was still present after flexible docking of the arylindazole 6 using FLO+ computational methodology (Thistlesoft) (Fig. 1C). This methodology imposed hydrogen bonding restraints to guide the central alcohol toward Asn-564 and the pyrazole N-2 toward Gln-570 but allowed flexibility of all side chains lining the binding pocket, including side chains forming the channel itself. The new channel is seen to expand away from the meta position of the N-phenyl ring of 6 (Fig. 1C), and an exploration of this “meta” channel was undertaken in the search for additional receptor interactions that might deliver increased potency and/or selectivity. A meta-carboxylic acid synthon 9 was chosen to allow rapid exploration of these interactions through reactions with suitable amines to give a series of meta-amide derivatives (Scheme 2). The selection of amines was carried out using a 3-stage process involving AlleGrow computational growth of groups into the channel, followed by FLO+ scoring to eliminate those showing least complementarity to the site, and finally close visual examination of the virtual products within the channel to select those showing the most promising interactions. The meta-amide growth start point used for AlleGrow placed the amide manually in the 2 possible planar orientations relative to the phenyl ring. AlleGrow uses growth through incremental addition of atomic or small molecular fragments, with exploration of conformational space after each addition and finally evaluation of complementarity with the surrounding protein residues. For this work AlleGrow was run multiple times, setting at each occasion a different minimum number for the atom growth from 4 to 10 atoms and requesting 1,000 solutions for each run. In total AlleGrow proposed more than 7,000 compounds displaying a variety of interactions with the channel: hydrophilic, hydrophobic, and mixed. In particular, residues Lys-667, Glu-540, Arg-611, Tyr-663, and Gln-570 were used for hydrogen bonding interactions, whereas Leu-603 and Ala-573 were used for hydrophobic interactions.

A small series of mainly polar amides designed using this process was prepared, with many showing potent GR agonist activity (24). One compound in particular, the D-alaninamide 10 was found to be 10-fold more potent than the unsubstituted parent 6 in the NF-κB TR assay. In common with the parent 6 and conventional corticosteroids, compound 10 displays full efficacy (~106% response relative to the standard dexamethasone, 100%) in this TR assay and in a mouse mammary tumor virus long terminal repeat (MMTV) reporter assay of TA (~130% response) (Table 1). This remarkable potency makes the nonsteroidal agonist 10 comparable to the most potent corticosteroids, such as fluticasone furoate 2. It was noted that AlleGrow predicted favorable interactions between the alaninamide moiety and 2 residues (Lys-667 and Glu-540) in the “meta” channel (Fig. 1D). Interestingly, introduction of the alaninamide substituent also enhanced PR agonist activity, with compound 10 showing inferior GR/PR selectivity to the unsubstituted parent 6 (Table 1). This enhancement of PR activity on introduction of the meta-amide substituent is a noteworthy observation and indicates that a similar “meta” channel must also open up in the PR.

It was discovered that PR agonist activity could be reduced by substitution of the secondary amide NH to give tertiary amide derivatives. Thus, constraining the D-alaninamide in a 5-membered ring gave the D-prolinamide 11, which showed only slightly lower GR potency than 10 but significantly reduced PR activity, resulting in GR/PR selectivity of >100-fold. In addition, compound 11 shows excellent selectivity over other steroid receptors, with no significant activity (pXC50 <5.6) seen in in vitro assays of androgen receptor (AR) and mineralocorticoid receptor (MR) agonism, MR antagonism, and AR and estrogen receptor (ERα and -β) binding. Compound 11 was also shown to display very low oral bioavailability (rat 1.5%, dog 5%), offering the ideal pharmacologic and pharmacokinetic profile for intranasal/inhaled development. Furthermore, whereas the highly insoluble steroidal glucocorticoids need to be delivered as intranasal suspensions, the more flexible nonsteroidal ligand 11, with its polar prolinamide functionality, was shown to possess sufficient aqueous solubility to allow formulation as an intranasal solution (24).

The design cycle was closed with the crystallographic determination of compound 11 in the GR LBD at a resolution of 3.0 Å. As before (7), crystallization was achieved in the presence of a 12-residue TIF-2 coactivator peptide. The x-ray structure (Fig. 2) demonstrated clearly the expected interactions and the success of the design strategy. Thus, the structure confirmed the ability of the arylindazole to expand the GR binding pocket in the same way as cortivazol and showed good agreement with the binding mode predicted from the modeling studies. Thus, movement of the “charge clamp” residues Gln-570 and Arg-611...
provides this increased volume and opens up access to the “meta” channel. Compound 11 is found to extend 7 to 8 Å into the “meta” channel, with Gln-570 forming a hydrogen bond to the indazole nitrogen (as predicted) and Arg-611 now interacting with the prolinamide group. The prolinamide group can also be seen to hydrogen bond to Glu-540, an interaction predicted by AlleGrow for the alaninamide (Fig. 1D). The remainder of the prolinamide group neatly occupies the “meta” channel. Within the classic binding pocket, the typical alcohol interaction with Asn-564 is present, and positioning of the 2-methoxy-5-fluoroaryl group in a favorable edge–face interaction with Tyr-735 is revealed, whereas the indazole 6-methyl group is accommodated in a small pocket at the center rear of this representation.

The high-potency meta-amides 10 and 11, like their unsubstituted parent 6, show full efficacy for both TR and TA (Table 1). However, further elaboration of the meta-amide substituent has provided evidence that the intrinsic pharmacology around this template can also be manipulated via the “meta” channel. Thus the (3S)-2-pyrrolidinone amide 12, although less potent than the D-prolinamide 11, retains much of the desired TR activity, inhibiting TNF-induced NF-κB activation by ~79% (compared with dexamethasone). However, this compound shows a greatly reduced TA activity, inducing only a quarter of the MMTV with dexamethasone). However, this compound shows a greatly reduced TA activity, inducing only a quarter of the MMTV with dexamethasone). This suggests that cofactor interactions are not affected by the “meta” channel extensions and that a mechanism other than disruption of the AF2 surface must be driving the reduced efficacy observed with compound 12.

Having gained additional interactions with the receptor through this unique channel, we hypothesized that this might reduce the necessity for some of the interactions in the traditional binding pocket and provide simpler ligands more compatible with oral delivery. To test this hypothesis we explored a series of truncated compounds retaining the favored D-alaninamide substituent. One such compound, the bis-trifluoromethyl analogue 13, was indeed found to retain potent GR agonist activity (NF-κB pIC50 8.9) and good GR/PR selectivity (Table 2). Although very much less potent than the larger D-alaninamide 11, the truncated analogue 13 still displays in vitro potency superior to the gold standard oral corticosteroid prednisolone and retains full TR and TA activities (Table 2). This result demonstrates the potential of “meta” channel occupation to deliver novel GR agonists in which the traditional ligand pocket is now only partially occupied. Interestingly, the...
corresponding D-prolinamide analogue 14 retains GR agonist activity (NF-xB pIC50 7.4), but there is a bigger drop-off in potency than between 10 and 11. Encouragingly, this more Lipinski-compliant (26) truncated analogue 14 (M, 517, cLogP 3.15) now shows measurable oral bioavailability of 10.5% in the rat, confirming the potential of this strategy to deliver novel oral nonsteroidal glucocorticoids. As in the full-length series, preliminary evidence for modulation of the pharmacologic profile by manipulation within the “meta” channel is also observed in the truncates. Thus compound 14 shows evidence for a partial agonist response, with reduced efficacy for both TR (≈89%) and TA (≈73%), whereas the simple meta-acetamide derivative 15 shows no agonist activity in either assay. Compound 15 clearly demonstrated the ability to displace labeled dexamethasone in an in vitro GR binding assay, and indeed was shown to antagonize the ability of dexamethasone to transactivate the MMTV reporter gene (pIC50 5.8 ± 0.1; maximum response 102% relative to RU-486).

Crystals of the truncated alaninamide 13 GR LBD/TIF-2 complex were obtained that provided a 2.5 Å resolution crystal structure (Fig. 2B), which again confirmed the expected occupation of the “meta” channel. The alaninamide amide group makes alternative H-bonding interactions to those seen for the prolinamide 11. Thus, hydrogen bonds are now seen to be made to Lys-667 via a water bridge and also to the backbone carbonyl of Gln-570. The indazole nitrogen is once again seen to be within H-bonding distance to Gln-570, and the usual hydrogen bond from the alcohol to Asn-564 is again evident. As expected, the truncated structure does not reach the region of the receptor occupied by the D-ring and 17α ester of fluticasone furoate 2 and the 2-methoxy-5-fluorophenyl group of compound 11. Interestingly, a molecule of the crystallographic adduct β-hexylglucoside is incorporated in the crystal structure. This can be seen to sit with the carbohydrate portion on the outside of the receptor and the hexane chain pushing into the unoccupied 17α pocket (Fig. 2B).

**Table 2. In vitro GR and PR activities for truncated indazoles 13–15, compared with prednisolone**

<table>
<thead>
<tr>
<th>Compound</th>
<th>GR binding pIC50*</th>
<th>GR NF-xB pIC50 (% max)†</th>
<th>GR MMTV pIC50 (% max)†</th>
<th>PR pEC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>7.9 ± 0.1 (n = 3)</td>
<td>8.9 ± 0.2 (n = 8)</td>
<td>7.3 ± 0.1 (n = 7)</td>
<td>6.5 ± 0.2 (n = 6)</td>
</tr>
<tr>
<td>14</td>
<td>7.4 ± 0.3 (n = 3)</td>
<td>7.4 ± 0.2 (n = 6)</td>
<td>6.3 ± 0.2 (n = 4)</td>
<td>&lt;5 (n = 2)</td>
</tr>
<tr>
<td>15</td>
<td>6.9 (n = 1)</td>
<td>&lt;-5.2 (n = 2)</td>
<td>&lt;-5.2 (n = 2)</td>
<td>&lt;5 (n = 2)</td>
</tr>
<tr>
<td>Prednisolone</td>
<td>7.7 ± 0.1 (n = 4)</td>
<td>8.0 ± 0.1 (n = 5)</td>
<td>7.3 ± 0.4 (n = 5)</td>
<td>&lt;5 (n = 7)</td>
</tr>
</tbody>
</table>

*Assay format B: pIC50 values = 0.5 lower than for assay format A.
†Maximum response relative to dexamethasone (100%).

Discussion

Computational modeling of the GR revealed the existence of a previously unexplored channel extending away from the typical steroidal A-ring region of the GR ligand-binding pocket, and this channel has been exploited for drug design on a nonsteroidal indazole template with the aid of an automated virtual design tool, AlleGrow. A series of amides were designed and prepared to rapidly explore the occupation of this channel, and the D-alaninamide 10 was found to show remarkable GR agonist potency comparable to that seen with the most potent corticosteroids. This result clearly confirms the potential of the “meta” channel to deliver enhanced GR potency. However, compound 10 also shows enhanced PR agonist activity, but this could be tempered, for example, by constraining the D-alaninamide to give the corresponding D-prolinamide 11. Compound 11 displays a very attractive potency, selectivity, and pharmacokinetic profile for topical delivery. Furthermore, unlike potent steroidal GR agonists, compound 11 displays sufficient aqueous solubility to allow delivery as an intranasal solution, which may offer enhanced efficacy in rhinitis at a reduced dose and more rapid onset of action. X-ray crystal structure determination of compound 11 in the GR LBD confirmed the existence and occupation of the “meta” channel and thereby validated the modeling and design methodology used.

The enhanced PR agonist activity seen with the D-alaninamide 10 compared with the unsubstituted parent 6 clearly suggests the presence of a similar channel in the PR. This is an important observation that could provide new opportunities in the design of modulators of the PR.

A major challenge of current nonsteroidal glucocorticoid research is the discovery of novel oral agents for the treatment of systemic inflammatory diseases, such as rheumatoid arthritis. Although the high molecular weight and high lipophilicity of compounds such as 11 (M, 665, cLogP 5.7) are acceptable for topical indications, these are clearly not suitable for oral delivery. However, occupation of the “meta” channel offers the prospect of simpler GR ligands by removing the reliance on interactions in the traditional steroid-binding pocket, as illustrated by the potent GR agonist activity seen with the truncated D-alaninamide derivative 13. This compound was designed to leave unoccupied a large proportion of the typical binding pocket in favor of “meta” channel occupation. Crystal structure determination of the receptor–ligand complex for compound 13 confirmed this binding mode and demonstrated clearly that full occupation of the typical steroidal binding pocket is not essential for potent agonist activity. This result offers the prospect of simplified GR ligands with physicochemical properties more compatible with oral administration, and an early indicator of this opportunity is provided by the modest oral bioavailability seen in the rat with the truncated D-prolinamide 14. Whereas the potent analogues 10, 11, and 13 show full TR and TA activities, data with the less-potent (3S)-2-pyrrolidinone amide 12 clearly demonstrate the potential for further elaboration of the meta-substituent to deliver novel glucocorticoid pharmacology, and this area is continuing to be investigated. Finally, the chance incorporation of β-hexylglucoside in the crystal structure of the compound 13 has revealed yet another channel away from the steroidal ligand-binding pocket, this time via the 17α pocket, which could offer further possibilities for novel GR ligand design.

In summary, x-ray crystal structures and molecular modeling have been used to design highly potent nonsteroidal glucocorticoids. Occupation of the “meta” channel has been confirmed through crystallographic determination of GR LBD complexes with the amide derivatives 11 and 13. The highly potent and selective D-prolinamide analogue 11 displays properties compatible with development as an intranasal solution formulation, whereas the truncated D-alaninamide derivative 13 demonstrates activation of the receptor with only partial occupation of
the steroid-binding pocket. Finally, data with the pyrrolidine amide 12 show the potential to separate TR and TA activities via further exploration within the “meta” channel. The discovery of these additional interactions with the GR offers significant opportunities for the design of novel GR modulators, including orally bioavailable analogues.

Materials and Methods

Synthesis. The meta-amide derivatives 6 and 10-12 were prepared from 2-[(5-fluoro-2-[methyl oxy]phenyl)-2-methylpropyl]-2-(trifluoromethyl)oxirane, whereas the truncated analogues 13-15 were derived from 2,2-bis(trifluoromethyl)oxirane (Scheme SI). For details, see SI Materials and Methods.

Crystallographic Methods. The GR LBD protein was the same F602Y and C638G construct used for fluticasone furoate and was purified and complexed with ethyl)oxirane (Scheme S1). For details, see Biggadike et al. PNAS

SI Materials and Methods. The GR Binding Assay. The ability of compounds to bind to the GR was determined by assessing their ability to compete with either an Alexa 555 fluorescently labeled (assay format A) or a Cy3b fluorescently labeled (assay format B) dexamethasone. For details, see SI Materials and Methods.

GR-Mediated Transrepression of NF-κB Activity. Human AS49 lung epithelial cells were engineered to contain a secreted placent al alkaline phosphatase gene under the control of the distal region of the NF-κB-dependent endothe lium–leukocyte adhesion molecule (ELAM) promoter, as previously described by Ray et al. (27). Incubation with test compounds for 16 h was followed by measurement of alkaline phosphatase activity. Dose–response curves were constructed, from which pIC50 values were estimated and from which maximal responses are calculated relative to dexamethasone (100%). For details, see SI Materials and Methods.


Glucocorticoid-Mediated Gene Transactivation: MMTV Assay. Human AS49 lung epithelial cells were engineered to contain a Renilla luciferase reporter gene construct under the control of MMTV. Incubation with test compounds for 6 h was followed by measurement of luciferase activity. Dose–response curves were constructed, from which pEC50 values were estimated and from which maximal responses were calculated relative to dexamethasone (100%).

Assay for PR Agonist Activity. A T225 flask of CV-1 cells at a density of 80% confluence was washed briefly with PBS, detached from the flask using 0.25% trypsin, and counted using a Sysmex KX-21N. Cells were diluted in DMEM containing 5% HyClone and 2 mM L-glutamine and transduced with 100 multiplicity of infection (MOI) of PrB-BacMam and 100 MOI of MMTV-BacMam. Cells were either plated in a flask for 24 h and then frozen or processed immediately. In either case, ~6 × 10^5 cells were dispensed to each well of a white well Nunc 384-well plate, containing compounds at the required concentration. After 24 h, 10 μL of Steady-Glo was added to each well of the plates. Plates were incubated in the dark for 10 min before being read on a ViewLux reader. Dose–response curves were constructed, from which pEC50 values were estimated.

Details of glucocorticoid antagonism and androgen, mineralocorticoid, and estrogen assays, as well as the peptide binding assay, are provided in SI Materials and Methods.

Pharmacokinetic Studies. Pharmacokinetic studies were conducted in GlaxoSmithKline laboratories in the United Kingdom under licence restrictions imposed by the United Kingdom Home Office. Rat studies were performed using adult male Sprague-Dawley rats, and studies in the dog used adult male beagles. For details, see SI Materials and Methods.

Computational Methods. All molecular modeling images were prepared using SYBYL version 7.3 (Tripos). Two computational methods, AlleeGrow and FLO++, were used to assist the design process. For details, see SI Materials and Methods.

Acknowledgments. We thank the following for their valuable contributions to this work: David Brown, Matilde Caivano, Margaret Clackers, Anette Miles-Williams, and Rosemary Sasse (screening and compound profiling); Bhavesh Patel, Heather Barnett, Karl Collins, and Natalie Wellaway (chemistry); James Gray, David Lugo, and Cesar Ramirez-Molina (drug metabolism); Eugene Stewart (computational chemistry); and Eric Hortense and Steve Jackson (analytical chemistry).

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**Supporting Information**

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**SI Materials and Methods**

**Synthetic Chemistry Experimental Methods. Chromatographic purification.** Chromatographic purification was performed using packed silica gel cartridges. The Flashmaster (Argonaut Technologies) is an automated multiuser flash chromatography system that uses disposable, normal-phase, solid-phase extraction cartridges (2 g to 100 g). It provides quaternary on-line solvent mixing to enable gradient methods to be run. Samples are queued using the multifunctional open access software, which manages solvents, flow rates, gradient profile, and collection conditions. The system is equipped with a Knauer variable-wavelength UV detector and 2 Gilson FC204 fraction collectors, enabling automated peak cutting, collection, and tracking.

**NMR.** 1H NMR spectra were recorded in either CDC13 or DMSO-d6 on either a Bruker DPX 400 or Bruker Avance DRX or Varian Unity 400 spectrometer, all working at 400 MHz. The internal standard used was either tetramethylsilane or the residual protonated solvent at 7.25 ppm for CDC13 or 2.50 ppm for DMSO-d6.

**Mass-directed autoreparative HPLC.** Mass-directed autoreparative HPLC was performed with Agilent 1100 Series LC/MSD hardware, using electrospray positive mode and running ChemStation 32 purification software. Column: Zorbax Eclipse XDB-C18 prep HT (dimensions, 212 × 100 mm, 5-μm packing), 20 mL/min solvent speed. Aqueous solvent was water plus 0.1% TFA. Organic solvent was MeCN plus 0.1% TFA. Specific gradient used: 1 min 70% water (0.1% TFA)/30% MeCN (0.1% TFA), increasing over 9 min to 5% water (0.1% TFA)/95% MeCN (0.1% TFA) to elute compounds.

**Liquid chromatography/mass spectrometry system.** The liquid chromatography/mass spectrometry (LC/MS) system used was as follows: column, 3.3 mm × 4.6 mm internal diameter, 3 μm ABZ+PLUS from Supelco; flow rate, 3 mL/min; injection volume, 5 μL; room temperature; UV detection range, 215–330 nm; detector A, 0.1% formic acid plus 10 mM ammonium acetate; and detector B, 95% acetonitrile + 0.05% formic acid. Gradients are shown in Table S1.

**Differential scanning calorimetry.** The differential scanning calorimetry (DSC) thermogram of crystalline 1-[(3S)-[(2R)-4-[5-fluoro-2-(methoxy)phenyl]-2-hydroxy-4-methyl-2-(trifluoromethyl)pentyl]amino]-6-methyl-1H-indazol-1-yl]phenylcarbonyl]D-prolinamide was obtained using a TA Q1000 calorimeter, serial no. 1000–0126. The sample was weighed into an aluminium pan and a pan lid placed on top and lightly crimped without sealing the pan. The experiment was conducted using a heating rate of 10 °C min⁻¹.

**Synthetic schemes.** The meta-amine derivatives 6 and 10–12 were readily prepared from the racemic epoxide 16 (1) (Scheme S1). Thus, ytterbium triflate–catalyzed opening of this epoxide with the amino indazole benzyl ester 17, followed by removal of the benzyl protection by hydrogenolysis, afforded the key intermediate, racemic meta-carboxylic acid 9. Amide bond formation via O-(7-azabenzotriazol-1-yl)-N,N,N′,N′-tetramethyluronium hexafluorophosphate (HATU)–mediated coupling of 9 with the requisite amine, followed by separation of isomers by chiral HPLC, afforded the required meta-amides 10 and 11. GR agonist activity has been shown to reside predominantly in isomers having the R configuration at the hydroxy-bearing stereogenic center, and asymmetric syntheses have subsequently been developed (1). The (3S)-2-pyrroolidine amide 12 was tested as the *2R/S* mixture. The truncated analogues 13–15 were prepared in a similar manner, starting from the simple achiral epoxide 18.

**Phenylmethyl 3-[(4-amino-6-methyl-1H-indazol-1-yl)benzoyl] 6-Methyl-1H-indazol-4-amine hydrochloride [may be prepared using the methodology described by Davies (2); 0.5 g, 2.7 mmol], phenylmethyl 3-iodomobenzoyl (0.9 g, 2.6 mmol), copper (I) iodide (14 mg, 0.07 mmol), potassium carbonate (1.2 g, 8.68 mmol), and trans,N,N′-pentamethyl-1,2-cyclohexandiamine (20 mg, 0.14 mmol) were heated together in N,N-dimethylformamide (DMF) (15 mL) at reflux overnight. The mixture was poured into water (15 mL), and ethyl acetate was added to dissolve the resulting oil. The suspension was then filtered through Celite. The organic phase was separated, combined with a second ethyl acetate extract, washed successively with water and brine, and then dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (75 g) eluting with 1–5% gradient of ethyl acetate in dichloromethane (DCM) to give the title compound as a light-brown oil (0.3 g).

1H-NMR: (CDCl₃, 400 MHz) δ 8.46 (t, 1 H), 8.10 (s, 1 H), 8.06 (m, 1 H), 7.96 (m, 1 H), 7.61 (t, 1 H), 7.49 (m, 2 H), 7.42 (m, 2 H), 7.38 (m, 1 H), 6.96 (s, 1 H), 6.31 (s, 1 H), 5.44 (s, 2 H), 4.15 (m, 2 H), 2.42 (s, 3 H).

**Phenylmethyl 3-[(4S)-[4-[5-fluoro-2-(methoxy)phenyl]-2-hydroxy-4-methyl-2-(trifluoromethyl)pentyl]amino]-6-methyl-1H-indazol-1-yl]benzoyl] 6-Methyl-1H-indazol-4-amine hydroiodide [may be prepared using the methodology described by Davies (2); 0.5 g, 2.7 mmol], phenylmethyl 3-iodomobenzoyl (0.9 g, 2.6 mmol), copper (I) iodide (14 mg, 0.07 mmol), potassium carbonate (1.2 g, 8.68 mmol), and trans,N,N′-pentamethyl-1,2-cyclohexandiamine (20 mg, 0.14 mmol) were heated together in N,N-dimethylformamide (DMF) (15 mL) at reflux overnight. The mixture was poured into water (15 mL), and ethyl acetate was added to dissolve the resulting oil. The suspension was then filtered through Celite. The organic phase was separated, combined with a second ethyl acetate extract, washed successively with water and brine, and then dried over anhydrous sodium sulfate and evaporated under reduced pressure. The residue was purified by silica gel column chromatography (75 g) eluting with 1–5% gradient of ethyl acetate in dichloromethane (DCM) to give the title compound as a light-brown oil (0.3 g).
((4-[5-fluoro-2-(methyloxy)phenyl]-2-hydroxy-2,4-dimethyl-pentyl-amino)-6-methyl-1H-indazol-1-yl)benzoic acid (200 mg, 0.357 mmol) and HATU (135.7 mg, 0.357 mmol) in DMF (2.5 mL) and the solution stirred at room temperature for 5 min. Alanimide hydrochloride (111.2 mg, 0.893 mmol) was added and the mixture stirred at room temperature overnight and then partitioned between 2 M HCl (50 mL) and ethyl acetate (50 mL). The organic layer was separated, washed with aqueous sodium bicarbonate (50 mL), dried over anhydrous sodium sulfate, and evaporated to give crude product, which was purified by mass-directed autopreparation (system B) to give the title compound (95.8 mg).

LC/MS: \( t_{RET} = 3.65 \text{ min}; \) MH+ = 630.

This mixture of diastereomers was resolved by chiral HPLC on a 2 in × 20 cm Chiralpak AD column eluted with heptane/ethanol 2:8 with a flow rate of 75 mL/min to provide diastereomer A (42.4 mg) and diastereomer B (45.2 mg).

**Diastereomer A (compound 10).** Analytic chiral HPLC (25 × 0.46-cm Chiralpak AD column, heptane/ethanol 2:8 eluting at 1 mL/min): \( t_{RET} = 5.1 \text{ min.} \)

LC/MS: \( t_{RET} = 3.66 \text{ min}; \) MH+ = 630.

**Diastereomer B.** Analytic chiral HPLC (25 × 0.46-cm Chiralpak AD column, heptane/ethanol 2:8 eluting at 1 mL/min): \( t_{RET} = 10.9 \text{ min.} \)

LC/MS: \( t_{RET} = 3.63 \text{ min}; \) MH+ = 656.

A sample of this diastereomer from a similar preparation was recrystallized from toluene to give very fine needles, melting point onset (DSC) 114 °C.

**Diastereomer A (2R isomer, compound 11).** Analytic chiral HPLC (25 × 0.46-cm Chiralpak AD column, heptane/ethanol 1:9 eluting at 1 mL/min): \( t_{RET} = 8.4 \text{ min.} \)

LC/MS: \( t_{RET} = 3.63 \text{ min}; \) MH+ = 656.

A sample of this diastereomer from a similar preparation was recrystallized from toluene to give very fine needles, melting point onset (DSC) 114 °C.

**Diastereomer B (25 isomer).** Analytic chiral HPLC (25 × 0.46-cm Chiralpak AD column, heptane/ethanol 1:9 eluting at 1 mL/min): \( t_{RET} = 22.4 \text{ min.} \)

LC/MS: \( t_{RET} = 3.63 \text{ min}; \) MH+ = 656.

3-(4-[[4-[5-Fluoro-2-(methyloxy)phenyl]-2-hydroxy-4-methyl-2-(trifluoromethyl)phenyl]amino]-6-methyl-1H-indazol-1-yl)phenyl[carbonyl]-D-prolinamide. Prepared similarly to D-alaninamide (10) from 3-(4-[[4-[5-Fluoro-2-(methyloxy)phenyl]-2-hydroxy-2,4-dimethylpenta-amino]-6-methyl-1H-indazol-1-yl]benzoyl acid and D-prolinamide.

LC/MS: \( t_{RET} = 3.63 \text{ min}; \) MH+ = 656.

Of this mixture of diastereomers, 118.8 mg was resolved by chiral HPLC on a 2 in × 20 cm Chiralpak AD column eluted with heptane/ethanol 1:9 with a flow rate of 75 mL/min to provide diastereomer A (59 mg) and diastereomer B (61 mg).

**Diastereomer A (2R isomer, compound 11).** Analytic chiral HPLC (25 × 0.46-cm Chiralpak AD column, heptane/ethanol 1:9 eluting at 1 mL/min): \( t_{RET} = 8.4 \text{ min.} \)

LC/MS: \( t_{RET} = 3.63 \text{ min}; \) MH+ = 656.

A solution of HATU (0.892 g, 2.347 mmol) in DMF (0.1 mL) was added to a solution of 3-(6-methyl-4-[[3,3,3-trifluoro-2-hydroxy-2-(trifluoromethyl)propyl]amino]-1H-indazol-1-yl)benzamide (13) (54 mg, 0.1 mmol) in DMF (0.5 mL) and DIPEA (1.435 mL, 8.22 mmol) in DMF (0.1 mL) at atmospheric pressure using an H-cube apparatus. Evaporation of the solvent left the title compound (120 mg).

LC/MS: \( t_{RET} = 3.58 \text{ min}; \) MH+ = 448.

N-methyl-3-(6-methyl-4-[[3,3,3-trifluoro-2-hydroxy-2-(trifluoromethyl)propyl]amino]-1H-indazol-1-yl)benzamide (15). A solution of HATU (38.1 mg, 0.1 mmol) in DMF (0.1 mL) was added to a solution of 3-(6-methyl-4-[[3,3,3-trifluoro-2-hydroxy-2-(trifluoromethyl)propyl]amino]-1H-indazol-1-yl)benzamide (44.7 mg, 0.1 mmol) and DIPEA (30 μL, 0.3 mmol) in DMF (0.2 mL) and the mixture shaken for 5 min. The mixture was then added to a solution of methylamine (3.1 mg, 0.1 mmol) in DMF (0.2 mL), and after shaking for 5 min the mixture was left at room temperature overnight. The solvent was then removed on a vacuum centrifuge, and the residue was dissolved in 1:1 DMSO/methanol (0.5 mL) and purified by mass-directed autopreparation. Product-containing fractions were combined and evaporated to give the title compound (2.46 mg).

LC/MS: \( t_{RET} = 3.39 \text{ min}; \) MH+ = 461.

N-[[1R]-2-aminoo-1-methyl-2-oxoethyl]-3-(6-methyl-4-[[3,3,3-trifluoro-2-hydroxy-2-(trifluoromethyl)propyl]amino]-1H-indazol-1-yl)benzamide (13). To a solution of 3-(6-methyl-4-[[3,3,3-trifluoro-2-hydroxy-2-(trifluoromethyl)propyl]amino]-1H-indazol-1-yl)benzamide (44.7 mg, 0.1 mmol) and DIPEA (1.435 mL, 8.22 mmol) and D-alaninamide hydrochloride (0.322 g, 2.58 mmol) in DMF (8 mL) were added, and the reaction mixture was stirred at 22 °C for a further 17 h when LC/MS showed the reaction to be complete. The solvent was evaporated, and the residue was partitioned between water and DCM. The organic phase was washed with water, dried by passage through a hydrophilic frit, and the solvent was evaporated to give a yellow oil. This crude product was purified first by silica gel chromatography eluting with a 0–15% methanol–DCM gradient to give impure material, which was then repurified by silica gel chromatography using a 0–15% methanol (+1% triethylamine)–DCM gradient to give the title compound as a yellow gum (1.05 g).

LC/MS: \( t_{RET} = 3.17 \text{ min}; \) MH+ = 518.

1-[[3-(6-Methyl-4-[[3,3,3-trifluoro-2-hydroxy-2-(trifluoromethyl)propyl]amino]-1H-indazol-1-yl)phenyl][carbonyl]-D-prolinamide (14). Prepared similarly to compound (13) from 3-(6-methyl-4-[[3,3,3-trifluoro-2-hydroxy-2-(trifluoromethyl)propyl]amino]-1H-indazol-1-yl)benzoyl acid and D-prolinamide.

LC/MS: \( t_{RET} = 3.09 \text{ min}; \) MH+ = 544.
Pharmacokinetic Studies. Rat pharmacokinetic studies were performed at GlaxoSmithKline Medicines Research Center (Stevenage, Herts, United Kingdom) under the licence restrictions imposed by the United Kingdom Home Office. The pharmacokinetics of compounds 11 and 14 were studied using adult male Sprague-Dawley rats (Charles River Laboratories). The dose formulation for i.v. administration of compound 11 and for i.v. and oral administration of compound 14 was 10% DMSO/50% PEG 200/40% water (vol/vol) administered at 1 mg/kg. For oral administration of compound 11, the compound was formulated as a suspension in 1% DMSO/99% 0.5% (wt/vol) methyl cellulose (aq) administered at 1 mg/kg. Test articles were administered discretely to ≥2 rats and serial blood samples collected into heparin-coated tubes at time points up to 12 h after administration. Plasma samples were harvested and stored at -20 °C before analysis.

Dog pharmacokinetic studies were performed at GlaxoSmithKline, The Frythe (Welwyn, Herts, United Kingdom) under the licence restrictions imposed by the United Kingdom Home Office. Compound 11 was studied using adult male beagles sourced from within GlaxoSmithKline. Three male dogs received a 30-min i.v. infusion of compound 11 at a nominal dose level of 0.5 mg/kg, followed 1 week later by an oral dose at a nominal dose level of 0.5 mg/kg administered as a crossover design. The i.v. dose was formulated as a solution in 10% DMSO/50% PEG 200/40% water at a nominal dose concentration of 0.2 mg/mL. The oral dose was formulated as a suspension in 0.5% (wt/vol) methyl cellulose in water at a nominal concentration of 1 mg/mL. Serial blood samples were collected from the cephalic vein in to heparinized tubes up to 12 h after dose. Plasma samples were harvested and stored at -20 °C before analysis.

Plasma samples were extracted using protein precipitation with acetonitrile using a 96-well filter plate (Whatman). The samples were drawn through the 96-well filter plate using a vacuum and the filtered extracts collected and diluted. The sample extracts were analyzed by LC-MS/MS analysis. HPLC separation was performed on a HP1100 binary pump and vacuum degasser (Agilent Technologies), HTS PAL autosampler (LEAP Technologies), and column-switching valve (Valco Instruments) using a Luna C18 5 mm × 2.1 mm column, 5-μm particle size (Phenomenex) operating at 40 °C. Samples were eluted at 800 μL/min using a gradient mobile phase consisting of 0.1% formic acid (vol/vol) in water and 0.1% formic acid (vol/vol) in acetonitrile. Test article detection was performed on a Sciex API4000 mass spectrometer (Applied BioSystems) using TurboIonSpray. Plasma standard curves were generated by a Sciex API4000 mass spectrometer (Applied BioSystems) using (vol/vol) in acetonitrile. Test article detection was performed on 0.1% formic acid (vol/vol) in water and 0.1% formic acid.

Pharmacokinetic parameters were determined using an in-house-developed macro operating within Microsoft Excel. Calculations were performed using noncompartmental analysis and using standard formula.

Biological Assays. GR binding assay. The ability of compounds to bind to the GR was determined by assessing their ability to compete with either an Alexa 555 fluorescently labeled (assay format A) or a Cy3b fluoroscence labeled (assay format B) dexamethasone. Compounds were solvated and diluted in DMSO and transferred directly into assay plates. Fluorescent dexamethasone and a partially purified full-length GR were added to the plates, together with buffer components to stabilize the GR protein (Panvera peptide [assay format A] or in-house stabilization peptide [assay format B]) and incubated at room temperature for 2 h in the dark. Binding of each compound was assessed by analyzing the displacement of fluorescent ligand by measuring the decrease in fluorescence polarization signal from the mixture. An appropriate reader was used to assess the displacement, with filters set for 535 nM excitation and 590 nM emission. Dose–response curves were constructed, from which pIC50 values were estimated.

GR-mediated TR of NF-kB activity. Human A549 lung epithelial cells were engineered to contain a secreted placentalkaline phosphatase gene under the control of the distal region of the NF-κB–dependent ELAM promoter, as previously described by Ray et al. (3). Compounds were solvated and diluted in DMSO and transferred directly into assay plates, such that the final concentration of DMSO was 0.7%. After the addition of cells (10 × 10^6 per well), 384-well plates were incubated for 1 h before the addition of 3.2 ng/mL human recombinant TNFα. After continued incubation for 16 h, alkaline phosphatase activity was determined by measuring the change in optical density at 405 nM with time after the addition of 0.35 volumes of assay buffer (2 mg/mL p-nitrophenylphosphate dissolved in 1 M diethanolamine, 0.28 M NaCl, and 0.5 mM MgCl2). Dose–response curves were constructed, from which pIC50 values were estimated and from which maximal responses were calculated relative to dexamethasone (100%).

Glucocorticoid-mediated gene TA: MMTV assay. Human A549 lung epithelial cells were engineered to contain a Renilla luciferase reporter gene construct under the control of MMTV. MMTV sequences include glucocorticoid responsive elements, which confer GR dependence on luciferase gene expression as a surrogate of GR-dependent TA. Compounds were solvated and diluted in DMSO and transferred directly into assay plates, such that the final concentration of DMSO was 0.7%. A 70% confluent T225 flask of A549 MMTV cells was harvested and diluted to 0.16 × 10^6/mL. Cell solution (70 μL) was dispensed to each well of white tissue culture–treated Nunc 384-well plates, containing compound at the required concentration. Plates were incubated for 6 h at 37 °C, 95% humidity, and 5% CO2. After incubation, luciferase activity was quantified by measurement on the ViewLux imager, after the addition of 10 μL of Renilla luciferase substrate. Renilla luciferase was prepared according to the manufacturer’s guidelines. Dose–response curves were constructed, from which pEC50 values were estimated and from which maximal responses were calculated relative to dexamethasone (100%).

Assay for GR MMTV antagonist activity. Human A549 lung epithelial cells were engineered to contain a Renilla luciferase reporter gene construct under the control of MMTV. MMTV sequences include glucocorticoid responsive elements, which confer GR dependence on luciferase gene expression as a surrogate of GR-dependent TA. Compounds were solvated and diluted in DMSO and transferred directly into assay plates, such that the final concentration of DMSO was 0.7%. A 70% confluent T225 flask of A549 MMTV cells was harvested and diluted to 0.16 × 10^6/mL. An appropriate EC80 of cortisol was added to the cell solution. Cell solution (70 μL) was dispensed to each well of white tissue culture–treated Nunc 384-well plates, containing compound at the required concentration. Plates were incubated for 24 h at 37 °C, 95% humidity, and 5% CO2. After incubation, luciferase activity was quantified by measurement on the ViewLux imager, after the addition of 10 μL of Renilla luciferase substrate. Renilla luciferase was prepared according to the manufacturer’s guidelines. Dose–response curves were constructed, from which pIC50 values were estimated and from which maximal responses were calculated relative to mitofron (RU-486) (100%).

Assay for AR agonist activity. CV-1 cells were transiently transfected with Fugene-6 reagent according to the manufacturer’s protocol. Briefly, a T175 flask of CV-1 cells at a density of 80% confluence was transfected with 25 μg of mix DNA and 75 μL of Fugene-6. The DNA mix (1.4 μg pAR, 2.5 μg pMMTV luciferase, and 18.75 μg pBluescript) was incubated with Fugene-6 in 5 mL Opti-
MEM-1 for 30 min and then diluted up to 20 mL in transfection media (DMEM containing 1% HyClone, 2 mM L-glutamine, and 25 mM Heps) before addition to the cells. After 24 h, cells were washed with PBS, detached from the flask using 0.25% trypsin, and counted using a Sysmex KX-21N. Cells were either frozen or processed immediately. In either case, transfected cells were diluted in assay media (DMEM containing 5% HyClone and 2 mM L-glutamine), and 5 × 10^4 cells were dispensed to each well of Nunc 384-well plates, containing compounds at the required concentration. After 24 h, 10 µL of Steady-Glo was added to each well of the plates. Plates were incubated in the dark for 10 min before being read on a Viewlux reader. Dose–response curves were constructed, from which pEC_{50} values were estimated.

**Assay for AR binding activity.** Briefly, 175 nM MBP-hAR-LBD (in-house protein), 1 nM AR red Fluoroprobe (Invitrogen), and 2 mM DTT were dissolved and mixed in the AR binding buffer [50 mM Tris (pH 7.5), 100 mM ammonium sulfate, 20% glycerol, 3% xylitol, and 5 mM CHAPS]. Ten microliters of the mix was dispensed to each well of Greiner low-volume 384-well plates, containing compounds at the required concentration. The plates were spun for 1 min at 200 × g, covered to protect the reagents from light, and then incubated at room temperature for ~2 h. Plates were read on an Acquest using a 485-nm excitation filter, a 530-nm emission interference filter, and a 505-nm Dichroic mirror. Dose–response curves were constructed, from which pIC_{50} values were estimated.

**Assay for ERα binding activity.** Briefly, 4 nM ERα (in-house protein) and 1.5 mM Fluoromone EL-Red (Invitrogen) were dissolved and mixed in ER binding buffer [50 mM Mops (pH 7.5), 50 mM NaF, 2.5 mM CHAPS, and 5 mM DTT]. Ten microliters of the mix was dispensed to each well of Greiner low-volume 384-well plates, containing compounds at the required concentration. The plates were spun for 1 min at 200 × g, covered to protect the reagents from light, and then incubated at room temperature for 2 h. Plates were read on an Acquest using a 530–25-nm excitation and 580–10-nm emission interference filter and a 561-nm Dichroic mirror. Dose–response curves were constructed, from which pIC_{50} values were estimated.

**Assay for ERβ binding activity.** Briefly, 6 nM ERβ (in-house protein) and 1.5 mM Fluoromone EL-Red were dissolved and mixed in ER binding buffer [50 mM Mops (pH 7.5), 50 mM NaF, 2.5 mM CHAPS, and 5 mM DTT]. Ten microliters of the mix was dispensed to each well of Greiner low-volume 384-well plates, containing compounds at the required concentration. The plates were spun for 1 min at 200 × g, covered to protect the reagents from light, and then incubated at room temperature for ~2 h. Plates were read on an Acquest using a 530–25-nm excitation and 580–10-nm emission interference filter and a 561-nm Dichroic mirror. Dose–response curves were constructed, from which pIC_{50} values were estimated.

**Assay for MR agonist activity.** Monkey kidney CV-1 cells were transiently transfected with Fugene-6 reagent according to the manufacturer’s protocol. Briefly, a T225 flask of CV-1 cells at a density of 80% confluency was transfected with 40 µL of Steady-Glo was added to each well of the plates. Plates were incubated in the dark for 10 min before being read on a Viewlux reader. Dose–response curves were constructed, from which pEC_{50} values were estimated.

**Peptide binding assay.** N-terminally biotinylated NR Box peptides derived from PGC-1 (DGTKPPQQEAEPEPILKLKLAPANT), RIP140 (LERNNIKQAANNNLSLHLSKQTIP), CBP (ADPEKRRKIQQQQLVLLHAKQO), SRC-1 (LDASKKESKD-HQLLRYYLDDKDEKD), p300 (GISPPLKPTVSQQALQONL-LRTLSP), TIF-2 (DQGOSRLHDGKOTKLQLTTLTKSDO), and the related sequence from NCoR (GHSFADPSNLGLEDIIRKALMSG) were individually coupled to the avidin-coated fluorescently distinct microspheres. These were incubated with Alexa 532-labeled GR LBD (containing an F602S mutation to enhance stability) in the presence of 40 µM of each compound for 4 h and the binding of the protein to each peptide assessed using flow cytometry.

**Computational methods.** All molecular modeling images were pre- pared using SYBYL Version 7.3. Two computational methods, AlleGrow and FLO + molecular modeling systems, were used to assist the design process. The GR ligand-binding site protein model, previously described (4), was used as a starting point, with compound 6 placed into the site using the FLO + “dockmin” procedure (500 iterations), allowing flexibility for all residues lining the binding pocket and creating zero bonds between the central hydroxy group and the Asn-564 side chain carbonyl and also from the ligand pyrazole to Gln-570 side chain NH. The receptor-positioned compound 6 was modified to introduce a “meta” amide group with an AlleGrow growth point in the trans position filling 1 valency of the amide nitrogen. The amide group, with growth point, was rotated in the plane of the phenyl ring to provide both reasonable starting points. The “grow” facility of AlleGrow was used, with multiple runs directing growth with targets of 4, 5, 6, 7, 8, 9, and 10 atoms. Output from these separate runs was combined to produce several thousand virtual compounds. These were scored within the protein site using the FLO+ scoring accessed through the AlleGrow utility “postgrow.” This uses the FLO+ “dockmin+” minimization procedure, with all virtual ligands finally binned according to predicted binding affinity. Those in low-affinity bins were discarded, whereas the remainder were evaluated visually within the site to make a selection of suitable compounds for synthesis. Thirty compounds, for which the related amines were readily available, were identified for synthesis.

**Crystallographic methods.** Crystallographic parameters are shown in Table S2. The GR LBD protein was the same F602S and
C638G construct used for fluticasone furoate and was purified and complexed with a 12-residue TIF2 coactivator peptide using the published protocol (5). Compound 11 was crystallized in 28% PEG 5,000 monomethyl ether and 0.1 M Mes 6.5, and the crystals formed over 1 month. The well condition was the cryoprotectant solution for freezing. These crystals were hexagonal and small, with the longest edge being 20 μm. The initial crystals of GR LBD complexed with compound 13 were obtained from the following crystallization conditions: 0.1 M Mes 6.5 and 1.6 M magnesium sulfate. The crystals were optimized after a second preparation of protein to 2.0 M magnesium sulfate with 0.1 M Mes 6.5 and the additive β-hexylglucoside, and the crystals formed overnight. These crystals were quite large and reproducible, with the longest edge being 400 μm. Both data sets were reduced and scaled using HKL2000. The structure for compound 11 was solved by molecular replacement using Amore and refined using the program CNX and Quanta as the building program. The crystal structure for compound 13 was solved by molecular replacement using Phaser and refined with Refmac, and Coot was used as the building program. The crystal structures have been deposited in the RCSB as 3K23 for the D-prolinamide 11 and 3K22 for the D-alaninamide 13.

Scheme S1.  (a) Yb(OTf)$_3$, CH$_3$CN, 85-100°C. (b) H$_2$, Pd/C, EtOH or MeOH. (c) Amine, HATU, DIPEA, DMF, room temperature. (d) Isomer separation by chiral HPLC.
Table S1. Gradients

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